

Spirulina sp. LEB-18 CULTURE USING EFFLUENT FROM THE ANAEROBIC DIGESTION

J. A. Borges, G. M. Rosa, L. H. R. Meza, A. A. Henrard,
M. R. A. Z. Souza* and J. A. V. Costa

Biochemistry Engineering Laboratory, Chemistry and Foods School, Phone: +55 (53) 3233-8653,
Fax: +55 (53) 3233-8745, P.O. Box 474, Federal University of Rio Grande,
Zip Code 96203-900, Rio Grande - RS, Brazil.
E-mail: mrandrade@furg.br

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Abstract - The carbon source is the most expensive nutrient for *Spirulina* production; effluents from anaerobic digestion contain this nutrient in the form of HCO_3^- . The aim of this study was to assess the growth kinetics, composition and fatty acid profile of *Spirulina* sp. LEB-18 grown in standard Zarrouk medium (NaHCO_3 16.8 g L^{-1}) and in Zarrouk medium replaced with 20% (v/v) effluent with reduced concentrations of NaHCO_3 (5.3 and 2.8 g L^{-1}). The use of effluent and lower concentrations of HCO_3^- was found to be an alternative to reduce the costs of *Spirulina* production, because there were no significant differences in growth parameters (μ_{max} 0.324 - 0.354 d^{-1} ; P_{max} 0.280 - 0.297 g L^{-1} d^{-1}), in the different culture medium used. Lipids ranged between 4.9 and 5.0%; the media with effluent had higher levels of linoleic acid compared to the standard medium.

Keywords: Carbon source for *Spirulina*; Fatty acid profile; Microalga.

INTRODUCTION

Microalgae are phototrophic microorganisms, and include prokaryotic photosynthetic bacteria, called cyanobacteria (Tomaselli, 2004). These microorganisms have been investigated for their potential to enrich foods, biofixate carbon dioxide (Morais and Costa, 2007), treat domestic and industrial effluents (Valderrama *et al.*, 2002) and produce biofuels. The advantages of microalgal biofuels (compared with first and second generation fuels) are that they do not contribute to deforestation or excessive water consumption and do not compete with land that could be used for food production (Costa and Morais, 2011).

Spirulina is a spiral-shaped cyanobacteria. Because of its properties, such as high nutritional value and the presence of valuable biocompounds, like phycocyanin (Moraes *et al.*, 2011), it is currently one

of the most studied microalgae. It is usually commercially produced in open bioreactors of up to 0.5 hectares in size (Belay, 1997), using sunlight as the light source.

Biogas production from the anaerobic digestion of *Spirulina* biomass is facilitated by its high concentration of organic matter (Costa *et al.*, 2008). Anaerobic digestion of biomass produces an effluent, which contains important nutrients such as carbon, nitrogen and phosphorus, and these can be recovered for the production of microalgal biomass (Converti *et al.*, 2009).

The main nutrient required for *Spirulina* cultivation is carbon, because the cells contain about 50% (w/w) of this element. Thus, the carbon source is the most expensive component of *Spirulina* production. For autotrophic growth (which is more suitable for large-scale open cultivation) carbon can be provided as CO_2 , carbonate or bicarbonate. If

*To whom correspondence should be addressed

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bicarbonate is used, it represents 60% of the cost of nutrients (Alava *et al.*, 1997), which is one of the reasons for studying the effects of lowering the concentration of the carbon source and finding alternative sources of this nutrient, such as molasses (Andrade and Costa, 2008), residual CO₂ (Ferreira *et al.*, 2012) and synthetic CO₂ (Rosa *et al.*, 2011).

The concentration of dissolved inorganic carbon in anaerobic effluent is lower than the amount indicated in the formulation of standard culture media for production of *Spirulina* biomass, such as Zarrouk medium (Zarrouk, 1966). Furthermore, the effluent generally needs to be diluted because of the toxic effects of high concentrations of ammonia, requiring supplementation with carbon. There are reports of *Spirulina* cultures with the addition of 2 g L⁻¹ NaHCO₃ at 2% (v/v) of anaerobic effluent (Olguín *et al.*, 2001), 4.5 g L⁻¹ of NaHCO₃ in 20% (v/v) of anaerobic effluent (Chaiklahan *et al.*, 2010), 4-10 g L⁻¹ of NaHCO₃ at concentrations of 0 to 100% (v/v) of anaerobic effluent, with the best results found with 8 g L⁻¹ of NaHCO₃ and 10% (v/v) of effluent (Cheunbarn and Peerapornpisal, 2010). But Radmann *et al.* (2007) and Andrade *et al.* (2008) found higher yields for this microalga in diluted culture media, which creates the possibility of testing lower concentrations of carbon to complement the culture medium with effluent.

There are studies in the literature regarding the cultivation of microalgae, such as *Chlorella* sp. (Wang *et al.*, 2010), *Chlorella vulgaris* (Kumar *et al.*, 2010), *Scenedesmus* sp. (Park *et al.*, 2010), and a microalgae consortium (Singh *et al.*, 2011) in anaerobic effluents; but there are no studies on the cultivation of microalgae in effluent originating from the digestion of their own biomass. However, any changes in the conditions of microalga cultivation (for example, the source of nutrients or its concentration in the medium) may result in different kinetic responses and composition of biomass (Vonshak and Torzillo, 2004). Therefore, the aim of this study was to assess the growth, composition and fatty acid profile of *Spirulina* sp. LEB-18 cultivated in the effluent of the anaerobic digestion.

MATERIAL AND METHODS

Effluent of the Anaerobic Digestion

The effluent was collected from an anaerobic bioreactor (310 L) fed with dried *Spirulina* sp. LEB-18 biomass for biogas production. The anaerobic bioreactor was operated in sequential batch mode

with daily cycles of feed (7.0 g L⁻¹ biomass of *Spirulina* sp. LEB-18), reaction (6 h), decantation (18 h) and emptying of 10% of the volume. The effluent was collected daily and stored in polyethylene bottles kept in a freezer (-18 °C) until further use. After thawing and homogenization, the effluent was centrifuged for 30 min at 4700 xg (HITACHI CT6EL, Taiwan) for removal of solids, and characterized.

The effluent's pH was measured with a digital pH meter (QUIMIS Q400H, Brazil). The alkalinity was determined by potentiometric titration with HCl solution (APHA, 1998). The bicarbonate concentration was determined from the pH and alkalinity (Andrade *et al.*, 2008). The ammoniacal nitrogen level was measured using the Nessler method, and phosphate according to the ascorbic acid method (APHA, 1998) using the reagent *PhosVer 3 Phosphate Reagent Powder Pillow*.

Cultivation of *Spirulina* sp. LEB-18

Inoculum

Spirulina sp. LEB-18 isolated from the Mangueira Lagoon in Santa Vitória do Palmar, Southern Brazil (33°30'13"S; 53°08'59"W) was used as inoculum, adapted in Zarrouk medium (Zarrouk, 1966) and in Zarrouk medium replaced by 30% (v/v) of effluent.

Culture Media

Spirulina cultivation was carried out in standard Zarrouk medium (16.8 g L⁻¹ NaHCO₃), for comparison (Assay 1). For assays using the effluent and reduction in the concentration of the carbon source, the medium consisted of 20% (v/v) of effluent and 80% (v/v) Zarrouk medium with reduced concentration of carbon source (NaHCO₃): 5.3 g L⁻¹ (Assay 2) and 2.8 g L⁻¹ (Assay 3) (Table 1).

Bioreactors and Operating Conditions

The study used raceway-type open bioreactors made from transparent acrylic with a total volume of 6 L, working volume of 4 L, surface area of 0.08 m² and 0.05 m in height. The medium was mechanically stirred with a paddle wheel at 17 rpm. The bioreactors were placed in a greenhouse made of transparent film and the cultures were maintained for 19 days, in a semicontinuous (Reichert *et al.*, 2006) mode with cut-off cellular concentration of 1.0 g L⁻¹ and medium renewal rate of 50%.

Table 1: Composition of the culture media used in the experiments.

	Assay		
	1	2	3
Concentration of carbon source (g L ⁻¹ NaHCO ₃)	16.8	5.3	2.8
Effluent (% v/v)	0	20	20
Zarrouk medium* (% v/v)	100	80	80

*Composition of the Zarrouk culture medium (g L⁻¹): NaHCO₃: 16.8; K₂HPO₄: 0.50; NaNO₃: 2.50; K₂SO₄: 1.00; NaCl: 1.00; MgSO₄.7H₂O: 0.20; CaCl₂: 0.04; FeSO₄.7H₂O: 0.01; EDTA 0.08; 1 mL Solution A5 containing (g L⁻¹): H₃BO₃: 2.86; MnCl₂.4H₂O: 1.81; ZnSO₄.7H₂O: 0.222; CuSO₄.5H₂O: 0.079; Na₂MoO₄: 0.015; 1 mL Solution B6 containing (mg L⁻¹): NH₄VO₃: 22.96; KCr(SO₄)₂.12H₂O: 192; NiSO₄.6H₂O: 44.8; Na₂WO₄.2H₂O: 17.94; TiO₂: 12.15; Co(NO₃)₂.6H₂O: 43.98.

Analytical Determinations

The biomass concentration (g L⁻¹) was determined daily by measuring the optical density of the liquid medium in a spectrophotometer (FEMTO 700 Plus, Brazil) at 670 nm (Costa *et al.*, 2002); the pH was measured using a digital pH meter (Quimis Q400H, Brazil) and the alkalinity by potentiometric titration with HCl solution (APHA, 1998). The environment and the medium temperatures were measured with mercury thermometers.

Growth Parameters

By using the biomass concentration determined experimentally it was possible to obtain the maximum specific growth (μ_{max}) by exponential regression of the logarithmic phase of the growth curve (Bailey and Ollis, 1986); the volumetric productivity (P_V , g L⁻¹ d⁻¹) (Equation (1)), where X_t (g L⁻¹) is the biomass concentration at time t (d) and X_0 (g L⁻¹) is the initial biomass concentration at time t_0 (d) (Schmidell *et al.*, 2001); and the areal productivity P_A (g m⁻² d⁻¹) (Equation (2)), which is an important factor for biomass production on a large scale. In Equation (2), P_V is the volumetric productivity, and "h" (m) is the height of the column of liquid in the bioreactor.

$$P_V = \frac{(X_t - X_0)}{(t - t_0)} \quad (1)$$

$$P_A = P_V \cdot h \quad (2)$$

Characterization of the Produced Biomass

The microalgal biomass was recovered from the liquid medium by centrifugation for 20 min at 4700 xg (HITACHI CT6EL, Taiwan), washed with distilled water to remove salts from the medium, dried at 40 °C for 48 h with forced air circulation (Quimis Q314D242, Brazil) and milled for later characterization.

The concentrations of protein and ash of the biomass were determined according to the official methodology (AOAC, 2000). Lipids were determined by the Folch method and carbohydrates were calculated by difference. The elemental composition (CHN) was determined using an elemental analyzer (Perkin Elmer 2400, USA) with acetanilide as a standard. The fatty acid profile was determined by gas chromatography (Varian STAR 3400CX, USA).

Statistical Analysis

The effects caused by the use of different carbon sources on the growth and composition of produced biomass were analyzed by analysis of variance (ANOVA), using the Tukey test at $p \leq 0.05$ to assess the significant differences between the results.

RESULTS AND DISCUSSION

Characterization of the Anaerobic Effluent

Anaerobic effluents are mainly made up of inorganic compounds, and the presence and concentration of these compounds depends on the characteristics of the substrate and operating conditions of the process. The high-protein substrate (biomass of *Spirulina*) used in the anaerobic digestion resulted in a low C/N ratio in the effluent, (Table 2).

The values of pH, alkalinity and ammoniacal nitrogen level determined in the effluent (Table 2) were similar to those found by Chaiklahan *et al.* (2010) in the effluent from the anaerobic digestion of pig manure used for the cultivation of *Spirulina platensis* (pH 7.97, alkalinity 1,040 mg L⁻¹ CaCO₃ and ammoniacal nitrogen 144 mg L⁻¹ N-NH₄), while the concentration of phosphate found (Table 2) is equivalent to 32% of the concentration in the standard Zarrouk medium. The alkalinity in the effluent is mainly due to the presence of carbonates (CO₃²⁻) and bicarbonates (HCO₃⁻), which in cultures of microalgae are absorbed from their conversion into CO₂.

Table 2: Characterization of the effluent from the anaerobic digestion used for the cultivation of *Spirulina* sp. LEB-18.

	Value
pH	7.80 ± 0.19
Alkalinity (mg L ⁻¹ CaCO ₃)	1,427.13 ± 176.59
Bicarbonate (expressed as mg L ⁻¹ NaHCO ₃)	2,383.10 ± 301.42
Ammoniacal nitrogen (mg L ⁻¹ N-NH ₄ ⁺)	361.35 ± 61.01
Phosphate (mg L ⁻¹ PO ₄ ³⁻)	86.01 ± 3.17
C/N (w/w)	5.49
Organic carbon (mg L ⁻¹)	67.63 ± 0.99

Organic carbon was detected in the effluent at a low concentration (67.63 mg L⁻¹, Table 2), which is typical of anaerobic effluents. Dilution of the effluent (20%, v/v) in Zarrouk medium for the preparation of culture media resulted in 0.07 g L⁻¹ of organic carbon in the medium and, due to the great difference between the concentration of organic carbon and the concentrations of the inorganic carbon source used (NaHCO₃ 2.8, 5.3 and 16.8 g L⁻¹), the heterotrophic growth of microalgae was disregarded in this study. The ammoniacal nitrogen concentration in the effluent is the sum of NH₃ and NH₄⁺ species. The concentration of each species is dependent on the equilibrium constant of the reaction: NH₃ + H⁺ ↔ NH₄⁺, and the temperature and pH of the medium. The non-ionized form (NH₃), at high concentrations, may inhibit microalgal growth. This compound can be converted to nitrate and thus be consumed by microalgae. However, the absorption rate of ammoniacal nitrogen is usually lower than that of nitrate due to the toxicity of ammonia (Grobbelaar, 2004).

Microalgae assimilate inorganic carbon compounds (carbonates/bicarbonates), nitrogen and phosphorus, and are being increasingly studied as an alternative for the treatment of effluents from the anaerobic digestion. In studies on bioremediation of wastewater by cyanobacteria, Forlani *et al.* (2011) reported the possibility of removing 50% of the anticorrosive phosphate genera by *Spirulina* cultures. Park *et al.* (2010) observed similar growth of *Scenedesmus* sp. using ammonia and nitrate in the study for the removal of ammonia from the effluent of the anaerobic digestion (pH 8.4, alkalinity 5,562 mg L⁻¹ CaCO₃ and ammoniacal nitrogen 1,196 mg L⁻¹ N-NH₄⁺).

Growth of *Spirulina* sp. LEB-18

In both the culture in the standard medium (Figure 1 (a)) and those with effluent (Figures 1 (b) and 1 (c)), the microalga grew immediately after the

start of the experiments. There were no significant differences between the results of maximum specific growth rate either between the results of maximum productivity when the experiments were carried out in the standard medium or media containing effluent and reduced carbon concentration (Table 3).

By comparing the kinetics parameters of growth of *Spirulina* sp. (Table 3) with those obtained in other studies using anaerobic effluents and/or reduction in the concentration of the carbon source, it was found that the values of volumetric productivity P_V obtained in this study (0.280 g L⁻¹ d⁻¹) are greater. Andrade *et al.* (2008) obtained a volumetric productivity P_V of 0.145 g L⁻¹ d⁻¹ when they cultivated *Spirulina* sp. LEB-18 at 2.8 g L⁻¹ of bicarbonate. In the cultivation of the microalgae *Chlorella sorokiniana*, *Scenedesmus bijuga* and *Chlorella minutissima* in 6% (v/v) of effluent from chicken manure diluted in deionized water, a P_V of between 0.063-0.076 g L⁻¹ d⁻¹ was achieved (Singh *et al.*, 2011); *Spirulina* sp. grown in 2% (v/v) of anaerobic pig manure effluent had a P_V of 0.182 g L⁻¹ d⁻¹ (Olguín *et al.*, 1997); in the cultivation of different microalgae in municipal sewage for biofuel production, P_V values of between 0.120 and 0.275 g L⁻¹ d⁻¹ were achieved (Zhou *et al.*, 2011).

Areal productivity (P_A) obtained in this study was about 14.00 – 14.85 g m⁻² d⁻¹ which is lower than in previous studies. *Spirulina platensis* cultivated in semicontinuous mode in a medium containing 20% (v/v) of effluent, 4.5 g L⁻¹ of NaHCO₃ and 0.2 g L⁻¹ of urea produced a P_{Amax} of 19.9 g m⁻² d⁻¹ (Chaiklahan *et al.*, 2010). For the batch cultivation of *Spirulina platensis*, Mezzomo *et al.* (2010) used pig effluent and found a μ_{max} 0.415 d⁻¹ in medium with 8.5% (v/v) of effluent and 6.5 g L⁻¹ of NaHCO₃. Without using anaerobic effluents, the batch cultivation of *Spirulina platensis* in modified Schlösser medium (without NaHCO₃ and Na₂CO₃) and CO₂ as the sole carbon source, produced a P_{Amax} of 22.3 g m⁻² d⁻¹ and μ_{max} of 0.25 d⁻¹ (Binaghi *et al.*, 2003).

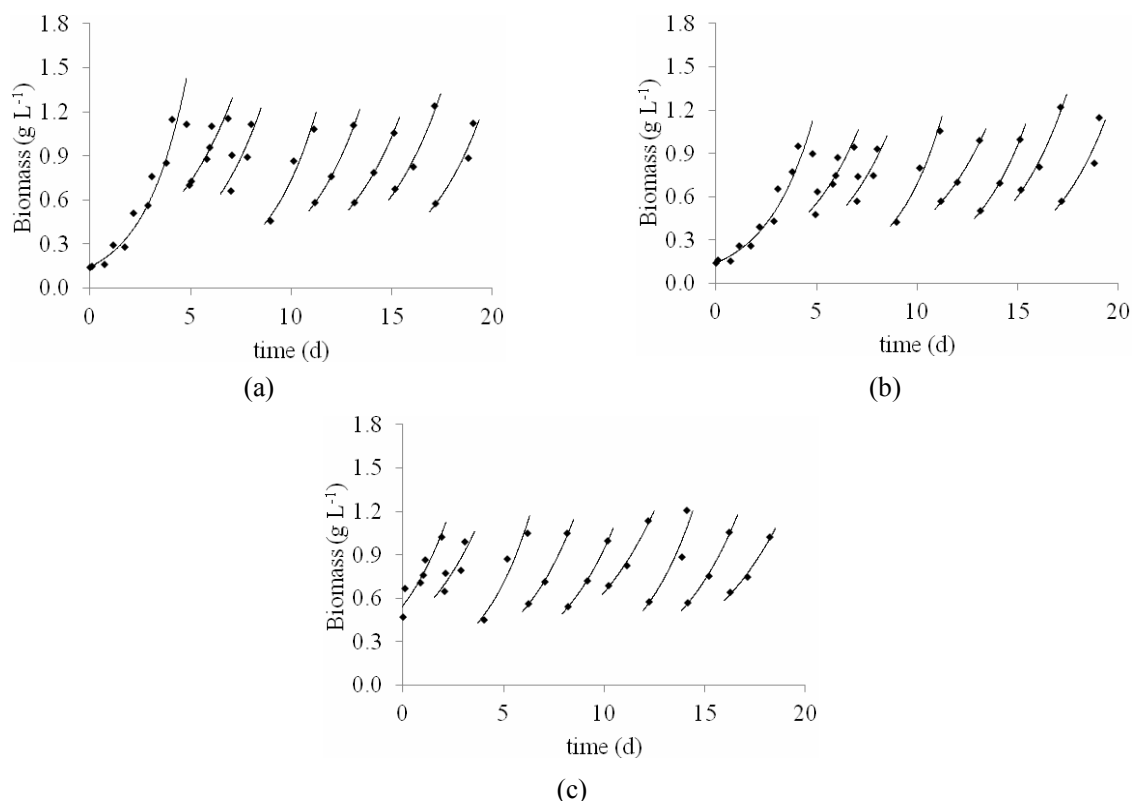


Figure 1: Growth curves of *Spirulina* sp. LEB-18 in: Standard Zarrouk medium (a), medium with 20% (v/v) of effluent and 5.3 g L⁻¹ NaHCO₃ (b), and medium with 20% (v/v) effluent and 2.8 g L⁻¹ NaHCO₃ (c).

Table 3: Kinetic parameters in cultures of *Spirulina* sp. LEB-18.

Assay	μ_{\max} (d ⁻¹)	$P_{V\max}$ (g L ⁻¹ d ⁻¹)	$P_{A\max}$ (g m ⁻² d ⁻¹)
1	0.325 ± 0.064 ^a	0.297 ± 0.071 ^a	14.85 ± 3.55 ^a
2	0.354 ± 0.060 ^a	0.280 ± 0.054 ^a	14.45 ± 2.70 ^a
3	0.324 ± 0.055 ^a	0.280 ± 0.064 ^a	14.00 ± 3.20 ^a

μ_{\max} : maximum specific growth rate; $P_{V\max}$: maximum volumetric productivity; $P_{A\max}$: maximum areal productivity. Assays 1: Cultivation in standard Zarrouk medium; 2: Cultivation in 20% (v/v) effluent medium and 5.3 g L⁻¹ NaHCO₃, and 3: cultivation in 20% (v/v) effluent medium and 2.8 g L⁻¹ of NaHCO₃. Equal superscript letters in the same column indicate that the results were not significantly different $p > 0.05$.

According to Lee (2001), areal productivities of 40 g m⁻² d⁻¹ have been found in experimental cultures, but $P_{A\max}$ between 20-25 g m⁻² d⁻¹ are usually reported in Raceway bioreactors. However, the height of the liquid column in the Raceway bioreactors used on an experimental scale (50 mm) is much smaller than the heights of the liquid column in reactors for large scale cultivation, which reach 400-500 mm, resulting in higher areal yields. In the results cited by Lee (2001), *Spirulina maxima* obtained a $P_{V\max}$ of 0.18 g L⁻¹ d⁻¹ (lower than the results found in this study), and a $P_{A\max}$ of 27 g m⁻² d⁻¹ (greater than the results found in this study).

The temperature and pH are factors that can influence the growth and composition of microalgae, in addition to the availability of nutrients, salinity

and light intensity (Hu, 2004). The pH values in cultures using effluent (Assays 2 and 3) had greater ranges of variation than the culture in standard medium (Assay 1), as seen in Figure 2(b). The environmental temperature varied between 26 and 44 °C, while temperatures of the liquid medium in the cultures varied between 25 and 37 °C, which covers the optimal range for the growth of *Spirulina* – between 30 and 35 °C (Tomaselli, 1997). Richmond and Grobbelaar (1986) reported that the optimum pH values for microalgae are between 9.5 and 10.5. These values are ratified by studies on the effects of these two factors on the production of *Spirulina*, in which there was a greater biomass concentration at pH 10.0 (Çelekli *et al.*, 2009) and 30 °C (Ogbona *et al.*, 2007). Rafiqul *et al.* (2005) also

studied the effects of these two factors, and found the greatest growth of *Spirulina platensis* was at pH 9.0 and a temperature of 32 °C.

Although the pH in the cultures with effluent (Assays 2 and 3) reached higher values than the optimum values found in the literature (9.5 to 10.5), the growth of microalga in these assays was similar to the assay with a standard culture medium. As the concentration of biomass increases (Figure 1), the bicarbonate concentration decreases (Figure 2 (a)), due to its consumption by the microalga, and the pH increases (Figure 2 (b)). This increase is because the consumption of carbon by microalgae is mainly from CO₂ which is the chemical specie with least resistance to diffusion across cell membranes (Giordano *et al.*, 2005; Miller and Colman, 1980). Because the chemical species of inorganic carbon in an aqueous medium play a role in the equilibrium $\text{CO}_{2(\text{aq})} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- \leftrightarrow \text{CO}_3^{2-}$, the consumption of CO₂ causes the system to react in the direction of its formation, consuming H⁺ ions from the medium and, consequently, raising the pH. Thus, the higher the photosynthetic activity, the greater the pH, as was observed in cultures with effluent and low carbon concentrations (Assays 2 and 3). The high pH values also imply lower losses of inorganic carbon into the

atmosphere, since at pH values above 10.5 the dominant chemical species in the equilibrium is CO₃²⁻, with a lower concentration of CO₂ in the medium and, therefore, a lower gradient of CO₂ concentration between the medium and the atmosphere, which promotes the loss of gas (Andrade *et al.*, 2008).

There were no differences in protein content in the biomass that was cultivated, which was above 60% in all experimental conditions (Table 4). Protein concentration can be up to 74% of dry weight of *Spirulina* (Cohen, 1997). In a study to remove the nutrients from wastewater by means of immobilized microalgae, Ruíz-Marín *et al.* (2010) reported an increase in protein content of the biomass of *Scenedesmus obliquus* cultivated in a semicontinuous mode (30%) when compared to microalgae biomass grown in a discontinuous mode (16%). The protein contents in the assays with effluent are higher than those found by Colla *et al.* (2007) (57.6%) and Rafiqul *et al.* (2005) (58.6%), who produced *Spirulina platensis* in Zarrouk medium and temperatures of 30 and 32 °C, respectively. However, Olguín *et al.* (2001) observed a 46% decrease in protein content of *Spirulina* sp. cultivated with 2% of the effluent of the anaerobic digestion when compared to the biomass produced in Zarrouk medium.

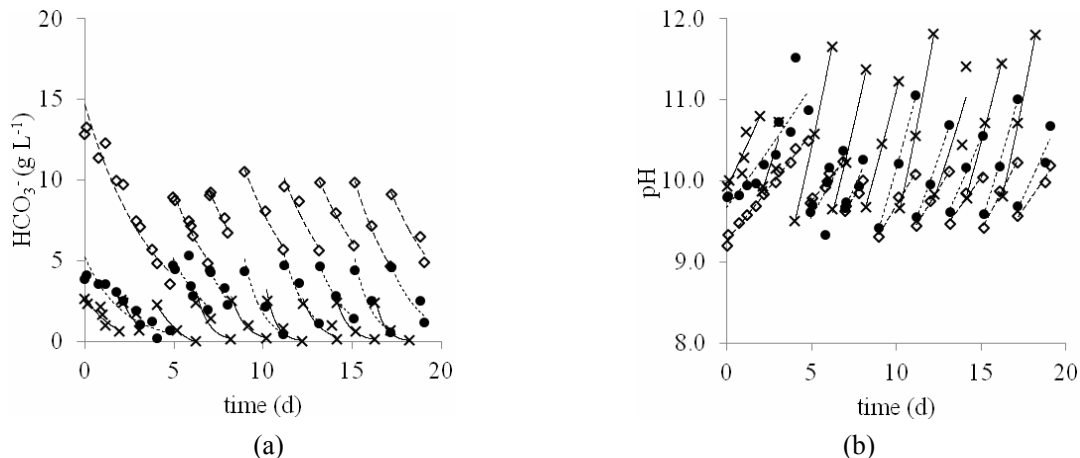


Figure 2: Variation of the concentration of HCO₃⁻ (a) and pH (b) of the cultures of *Spirulina* sp. LEB-18 in: Zarrouk medium (◇), medium with 20% (v/v) effluent and 5.3 g L⁻¹ NaHCO₃ (●), and medium with 20% (v/v) of effluent and 2.8 g L⁻¹ NaHCO₃ (x).

Table 4: Characterization of *Spirulina* sp. LEB-18 produced.

Assay	Proteins (%)	Lipids (%) [*]	Carbohydrates (%)	Ashes (%)	C (%) [*]	H (%) [*]	N (%) [*]
1	62.3 ± 0.8 ^{a,b}	4.9	21.6 ± 1.5	11.2 ± 1.2 ^b	45.3	9.4	9.8
2	63.6 ± 0.3 ^a	5.0	15.2 ± 0.4	16.2 ± 0.3 ^a	43.1	9.2	8.9
3	60.3 ± 2.6 ^b	5.0	19.1 ± 2.6	15.6 ± 0.2 ^a	41.5	8.7	8.7

1: Cultivation in Zarrouk medium, 2: Cultivation in 20% (v/v) effluent and 5.3 g L⁻¹ NaHCO₃, and 3: Cultivation in 20% (v/v) effluent and 2.8 g L⁻¹ NaHCO₃.^{*} Determination carried out without replicates. Equal superscript letters in the same column indicate that the results were not significantly different at p > 0.05.

Although the concentration of the carbon source in the cultures with effluent (Assays 2 and 3) fell to 31.6% and 16.7% of the concentration in the standard Zarrouk medium (Assay 1), the concentrations of elemental carbon in the biomass were 95.1% and 91.6%, respectively, of the concentration found in the biomass grown in Zarrouk culture medium (Table 4).

The high concentration of organic matter in the microalgal biomass (88.8% for Assay 1, 83.8% for Assay 2, and 84.4% for Assay 3) means that it can be used for biogas production, a process in which anaerobic micro-organisms decompose the organic fraction of biomass to produce CH₄ and CO₂. Another factor to be considered in anaerobic digestion is the C/N ratio, where appropriate values for the process are between 20 and 30 (Yang *et al.*, 2009). This ratio in the biomass produced varied from 4.62 (w/w) (Assay 1) to 4.84 (w/w) (Assay 2), and these values are characteristic of biomass with a high protein content. Even so, Costa *et al.* (2008), using *Spirulina* biomass with a C/N ratio of 4.37, produced biogas with 78% CH₄.

The lipid content (Table 4) was similar in all experimental conditions. The lipid content in *Spirulina* biomass at optimal conditions varies between 6 and 13% (Cohen, 1997) and can be manipulated by the concentration of nitrogen in the medium, temperature and salinity (Colla *et al.*, 2004). Olguín *et al.* (2001) produced *Spirulina* sp. in sea water supplemented with 2% (v/v) of pig manure wastewater and an illuminance of 66 μmol m⁻² s⁻¹, and obtained 28.6% lipids, while the cultivation in Zarrouk medium provided 8.0% of lipids.

The fatty acid profile of the biomass (Table 5) had a predominance of palmitic acid (C16:0) (37.77% for Assay 1, 38.48% for Assay 2, and 36.70% for Assay 3). In the three experimental conditions, the presence of linoleic fatty acids (C18:2c ω-6) and γ-linolenic acid (C18:3 ω-6) was detected and there were higher levels of linoleic acid in assays with effluent (Assays 2 and 3). Similar results were found by Olguín *et al.* (2001) who found 44.0% of palmitic acid in *Spirulina* sp. cultivated in Zarrouk medium and 43.4% in medium with effluent, as well as high proportions of γ-linolenic acid and a drop in this value in medium with effluent (28.1%) compared to the value found in Zarrouk medium (31.2%).

Spirulina is a known source of essential fatty acids, particularly γ-linolenic acid. In the assays with effluent (Assays 2 and 3), 19.6 and 18.7% of fatty acids were made up of the sum of linoleic and γ-linolenic fatty acids.

Table 5: Profile of fatty acids (%) of biomass of *Spirulina* sp. LEB-18 produced in Zarrouk medium (1), 20% (v/v) of effluent and 5.3 g L⁻¹ of NaHCO₃ (2) and 20% (v/v) of effluent and 2.8 g L⁻¹ of NaHCO₃ (3).

	Assay		
	1	2	3
Saturated fatty acids			
C 10:0	2.90	5.32	1.72
C 12:0	ND	ND	0.28
C 13:0	ND	ND	0.73
C 14:0	1.27	ND	1.48
C 15:0	0.33	ND	0.55
C 16:0	37.77	38.48	36.70
C 20:0	1.28	ND	0.50
Monounsaturated fatty acids			
C 14:1	ND	ND	0.22
C 15:1	1.86	ND	1.69
C 16:1	9.8	7.73	9.33
C 18:1c	1.01	3.36	1.90
C 18:1t	1.74	4.93	2.66
Polyunsaturated fatty acids			
C 18:2c (ω-6)	10.83	14.24	12.94
C 18:2t (ω-6)	7.24	12.88	12.10
C 18:3 (ω-6)	6.00	5.34	5.76
C 18:3 (ω-3)	11.97	5.78	7.53
C 20:2	1.77	ND	0.60
C 20:3 (ω-6)	0.86	ND	0.61
C 20:3 (ω-3)	1.25	ND	0.93
C 20:4 (ω-6)	ND	ND	0.29
C 22:2	1.67	1.93	1.47

C 10:0, decanoic acid (capric); C 12:0, dodecanoic acid (lauric); C 13:0, tridecanoic acid; C 14:0, tetradecanoic acid (myristic); C 15:0, pentadecanoic acid; C 16:0, hexadecanoic acid (palmitic); C 20:0, eicosanoic acid (arachidic); C 14:1, cis-9-tetradecaenoic (myristoleic); C 15:1, cis-9-pentadecaenoic; C 16:1, cis-9-hexadecaenoic (palmitoleic); C 18:1c, cis-9-octadecaenoic (oleic); C 18:1t, trans-9-octadecaenoic (elaidic); C 18:2cω-6, cis-9,cis-12-octadecadienoic (linoleic); C 18:2tω-6, trans-9,trans-12-octadecadienoic (linolelaidic); C 18:3ω-6, 6,9,12-octadecatrienoic acid (γ-linolenic); C 18:3ω-3, 9,12,15-octadecatrienoic (α-linolenic); C 20:2, 8,11-eicosadienoic acid; C 20:3ω-6, 5,8,11-eicosatrienoic; C20:3ω-3, 8,11,14-eicosatrienoic acid; C 20:4ω-6, 5,8,11,14-eicosatetraenoic (arachidonic); C 22:2, docosadienoic acid; ND: not detected.

In the group of ω-6 (omega-6) fatty acids, the main fatty acid is linoleic (C 18:2c), because mammals use this to synthesize other fatty acids such as γ-linolenic (C18:3 ω-6) and arachidonic (C20:4 ω-6). In the ω-3 (omega-3) group, the essential fatty acid responsible for the synthesis of other fatty acids with longer chains is α-linolenic acid (C18:3 ω-3). This bioprecursor of ω-3 fatty acids is present in fish, plants and algae, and in mammals it is the precursor of EPA (C20:5 ω-3) and DHA (C22:6 ω-3). α-linolenic acid was the fatty acid with the highest proportion in the biomass produced in Zarrouk medium (Assay 1) (Table 5). Arachidonic acid was detected (0.29%) in the *Spirulina* biomass produced from effluent and the lowest concentration of the carbon sources tested (2.8 g L⁻¹ NaHCO₃, Assay 3).

The proportions of omega-6 (ω -6) to omega-3 (ω -3) fatty acids found in the biomass were 2:1 (Assay 1), 6:1 (Assay 2) and 4:1 (Assay 3). According to the WHO (World Health Organization) and FAO (Food and Agriculture Organization) WHO/FAO (1995), the recommended ratio of ω -6: ω -3 for human nutrition is between 5:1 and 10:1.

The use of Zarrouk medium replaced by 20% effluent and 2.8 g L⁻¹ NaHCO₃ (Assay 3) resulted in a higher concentration of unsaturated (UFA, 58.03%) and polyunsaturated (PUFA, 42.23%) fatty acids. These results were higher than those found by Radmann and Costa (2008): 53.51% UFA and 29.37% of PUFA in the biomass of *Spirulina* sp. LEB-18 grown with CO₂, SO₂ and NO in tubular photobioreactors connected in series; and by Morais and Costa (2008): 37.50% UFA and 9.60% PUFA, when cultivating microalgae and replacing the standard carbon source (NaHCO₃) with 6% (v/v) of CO₂.

Although highly unsaturated fatty acids have a significant nutritional importance, their presence is undesirable for the production of biodiesel, for example, due to their high volatility, low oxidation stability and tendency to form gums (Halim *et al.*, 2011). Unlike most vegetable oils, microalgal oils generally contain higher levels of highly unsaturated fatty acids (with four or more double bonds), which have lower oxidative stability than unsaturated fatty acids with a smaller number of double bonds (such as linoleic and linolenic acids, for example) (Chisti, 2007). The biomass of *Spirulina* produced under different conditions had between 56.00% (Assay 1) and 58.03% (Assay 3) of unsaturated fatty acids in the profile. Only one unsaturated fatty acid with four double bonds was found (0.29% C 20:4 ω -6; assay 3).

In this study, the *Spirulina* biomass produced had a low lipid level (5.0%). Halim *et al.* (2011) assessed the fatty acid profile of the low-lipid (7.1 wt %) microalga *Chlorococcum* sp., and found C 18:1 (63%), C 16:0 (19%), C 18:2 (4%), C 16:1 (4%) and C 18:0 (3%). Others microalgae have a higher lipid content, such as *Scenedesmus obliquus* (58.3%) (Mandal and Mallick, 2009), *Choricystis minor* (60.0%) (Sobczuk and Chisti, 2010), *Nannochloropsis oculata* NCTU-3 (50.4%) (Chiu *et al.*, 2009) and *Chlorella protothecoides* (55.2%) (Xu *et al.*, 2006). In addition to the lipid content, other factors such as productivity, the ability to grow under specific environmental conditions, easy of recovery of the biomass from liquid medium and the composition of the oil should be considered when choosing a microalga for biodiesel production (Amaro *et al.*,

2011). The extraction of lipids from microalgae for biodiesel production has been widely studied, with a focus on reducing the costs of biomass production and manipulation of culture conditions to maximize lipid levels. Therefore, the use of anaerobic effluent is an alternative that can be evaluated to reduce the cost of nutrients for the production of *Spirulina*.

CONCLUSIONS

The reduction in the concentration of the carbon source (NaHCO₃) plus the supplementation of the medium with anaerobic effluent for the semicontinuous cultivation of *Spirulina* sp. LEB-18 is an alternative for obtaining biomass with growth kinetics and composition similar to those obtained in standard medium (Zarrouk).

The semicontinuous cultivation of *Spirulina* sp. LEB-18 with 20% (v/v) of anaerobic effluent and reduced concentration of carbon source (NaHCO₃ 2.8 and 5.3 g L⁻¹) resulted in maximum specific growth rates between 0.324 and 0.354 d⁻¹, volumetric productivity between 0.280 and 0.297 g L⁻¹ d⁻¹ and areal productivity between 14.00 and 14.85 g m⁻² d⁻¹, without significant differences between the different conditions studied. The biomass of *Spirulina* produced with effluent and the greatest reduction in carbon concentration (2.8 g L⁻¹ NaHCO₃) contained 5.0% lipids, a high protein level (60.3%) and a fatty acid profile of 41.96% saturated fatty acids, 42.23% polyunsaturated and 12.94% of the essential fatty acid linoleic acid.

The results obtained in this study corroborate the idea that it is possible to use the biomass produced from *Spirulina* as a source of biofuel, especially biogas, due to the high concentration of organic matter, and for the extraction of fatty acids. Although there is a high protein concentration in the biomass, its use as a pharmaceutical and food must await legislation, including compositional and microbiological analyses.

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